Crystal Violet Binding Assay for Assessment of Biofilm Formation by *Klebsiella pneumoniae* on Catheter, Glass and Stainless-steel Surfaces

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Abstract:
In this paper, quantified study of the biofilm formed by *Klebsiella pneumoniae* isolated from urine specimen of patient suffering from acute urinary tract infection (UTI) on catheter, stainless-steel and glass coupon surfaces, as well as determine the relationship between time contact and biofilm progression using crystal-violet binding assay based on the values of optical density at 620nm of the crystal violet stain which bonded total biofilm biomass by resolubizing with 99.9% ethanol at the specific interval times. The result showed biofilm formed on three tested surfaces but in different degrees. According to obtained data, the catheter coupons presents a higher capability to attract bacteria cell and biofilm formation followed by glass surfaces while stainless-steel surfaces regard as a less attractive surfaces in bacterial adhesion and biofilm progression. The attachment of the bacterial cells on the fresh produce surfaces increase with the contact time but the increase reached a maximum at time 48h. in which, the optical densities of catheter, glass and stainless-steel coupon surfaces were (0.169 nm), (0.085 nm) and (0.07 nm) respectively. The statical analysis showed significant differences between substratum type's adherence and biofilm progression.

Keyword: Biofilm, *Klebsiella pneumoniae*, Catheter, Glass, Stainless-steel, Crystal Violet Binding Assay.

أختبار ربط البلور البنفسجي لتقدير تكون الغشاء الحيوي لبكتريا *Klebsiella pneumoniae* على اسطح القثطار البولي و الزجاج و الفولاذ المقاوم للصدأ

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الخلاصة:
في هذا البحث، تمت دراسة كمية للغشاء الحيوي المتكون بواسطة بكتريا *Klebsiella pneumoniae* و المعزولة من عينة ادرار لمريض يعاني من التهاب المجاري البولية الحاد على كل من اسطح القثطار البولي و الزجاج و الفولاذ المقاوم للصدأ و تحديد العلاقة بين وقت تعرض القطع للعالق البكتيري و تطور الغشاء الحيوي للبكتريا ذاتها، بأستخدام أختبار ربط البلور البنفسجي و المعتمد على قيم الكثافة الضوئية لملون البلور البنفسجي المرتبط بالكتلة الحيوية الكلية للغشاء الحيوي و المستخلص بالكحول الايثانولي (99.9%) عند 620 نانوميتر ولفترات زمنية محددة . بينت النتائج تكون الغشاء الحيوي على الاسطح الثلاثة لكن بدرجات متفاوتة. وفقا للبيانات التي تم الحصول عليها، كان سطح القثطار البولي اكثر السطوح جذبا للخلايا البكتيرية و تكوينا للغشاء الحيوي يليه السطح الزجاجي بينما كان سطح الفولاذ المقاوم للصدأ الأقل جذبا للبكتريا، و فيها

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التصاق الخلايا البكتيرية للسطوح اعلاه يزداد بأزدياد وقت تعرضها للعالق البكتيري و ان هذة الزيادة تصل حدها الأعلى عند الساعة 48 حيث كانت الكثافة الضوئية لكل من سطح القثطار البولي و الزجاج و الفولاذ المقاوم للصدأ (0.169nm) و (0.085nm) و(0.07nm) على التوالي. أظهر التحليل الاحصائي فروق ذات دلالة إحصائية بين انواع اسطح الالتصاق و تطور الغشاء الحيوي.

Introduction:

Nearly 99% of microorganisms living on earth live in microbial communities known as biofilms [1]. The hospital environment is particularly susceptible to contamination by bacterial pathogens that grow on surfaces as biofilms. Most Gram-positive bacteria, such as *Enterococcus* spp. including Vancomycin-resistant *Enterococcus* (VRE), *Staphylococcus aureus* including Methicillin-resistant *Staphylococcus aureus* (MRSA), or *Streptococcus pyogenes* and Many Gram-negative species, such as *Acinetobacter* spp., *Escherichia coli*, *Klebsiella* spp., *Pseudomonas aeruginosa*, *Serratia marcescens*, or *Shigella* spp. can survive on inanimate surfaces even for months. These species were found among the most frequent isolates from patients with nosocomial infections [2]. *Klebsiella* spp., particularly *K. pneumoniae*, is a common hospital-acquired pathogen that causes nosocomial infections such as pneumonia (lung infections), wound infections, meningitis, abscesses, urinary tract infections and diarrhea [3].

Biofilm-associated cells could be differentiated from their suspended counterparts by generation of an extracellular polymeric substance (EPS) matrix, reduced growth rates, and the up- and down-regulation of specific genes. Biofilms have greater importance for public health because of their role in certain infectious diseases and importance in a variety of device-related infections [4].

Health care-associated infections (HAIs) remain a major cause of patient morbidity and mortality. Although the main source of nosocomial pathogens is likely the patient's endogenous flora, an estimated 20% to 40% of HAIs have been attributed to cross infection via the hands of health care personnel, who have become contaminated from direct contact with the patient or indirectly by touching contaminated environmental surfaces [5].

The importance of the biofilms to the clinician lies in the fact that they have been implicated in several common infectious processes, such as urinary tract infections, catheter infections, middle-ear infections, formation of dental plaque, gingivitis[6] coating contact lenses[7] and intrauterine devices[8]. Serious and lethal processes such as endocarditis, infections in cystic fibrosis and infections of permanent indwelling devices such as joint prostheses and heart valves may also be associated with biofilms [9]. Since them are resistant to bacteria, they tend to persist and provide a focus for further activation and spread of infection [10].

This study presents a quantitative evaluation of the adherence cells and the biofilm formed by *K. pneumoniae* on the three solid surfaces (catheter, glass and stainless-steel surfaces), that are used in manufacturing tools and medical devices, as well as the relationship between time contact and biofilm formation.

Materials and methods

Bacterial isolate and growth condition:

This study was conducted by *Klebsiella pneumoniae* maintained in BHI-slant at 4˚C, these isolate sub-cultured on BHI-broth at 37˚C for 24 h. and streaking on MacConkey agar before use. A single, isolated colony was grown in 50ml of BHI-broth that incubated with shaking at 37˚C for 18 h. these used in adherence study.

Test coupons

Stainless steel and glass surface coupons were used to study biofilm formation. These coupons were cut into uniform size of 1cm x 1cm while catheter was capped at both ends in 1 cm in length for the adhesion experiments. The first two types of coupons were sonicated using commercial detergent then rinsed in distilled water for 30 min. After that, they were thoroughly rinsed by immersion in boiling distilled water to remove any remaining detergent prior to use, and dried for 2h at 60ºC, then distributed in sterile tubes and sterilized in an autoclave at 121ºC for 15 min. [11-12], while catheters were cutting under sterile conditions.

Biofilm formation on the tested coupons

Working culture was containing 2 ml of the bacterial suspension BHI-broth each 18 ml of low nutrient medium BHI-broth (BHIB diluted ten times) [13]. The effect of substratum type of the tested surfaces was investigated by incubated of the broth medium at 37 ˚C and pH 7.

For inoculation, 2 ml of the standardized *K. pneumoniae* suspension was inoculated on the 1cm x 1 cm different coupons. For the negative controls, 2 ml of sterile low nutrient medium solution was used to substitute the 2 ml of *K. pneumoniae* suspension, and then these coupons incubated for 0, 6, 24, 48, 72h.

Biofilm quantification

Crystal violet assay was used to quantify the biofilm formation which measures the total biofilm biomass, including bacterial cells and extracellular polymeric substances (EPS) matrix. This assay was adapted from [12,14,15].

At the end of each incubation periods, a set of coupons were aseptically removed and rinsed three time using 1 ml distilled water. This step was used to rinse off loosely attached bacterial cells. Then, they were air dried and adherent bacteria were stained with 2 ml of 0.1% (w/v) crystal violet for each coupon at 28°C for 20 min. The exceed crystal violet solutions were removed from the coupons and rinsed with 1 ml distilled water thrice and air dried. After drying, the attached crystal violet was solubilized with 2 ml of 95% (v/v) ethanol for 20 min.; finally 200 µl of extracted stain was transferred to a clean Micro-titer plate for measuring by using ELISA auto-reader the wavelength at 620 nm.

The concentration of crystal violet was determined by measuring the optical density of destaining solution at 620 nm (CV-OD620 value). To correct the background staining, the mean CV-OD620 value obtained for the controls was subtracted from the mean CV-OD620 value obtained for *K. pneumoniae* on the different biotic coupons. This experiment conducted twice with duplicate in each time.

Results and discussion

In this study, biofilm formation by *K. pneumoniae* revealed that these bacteria possess a high capacity to form biofilm on the three surfaces used (Catheter, Stainless steel and Glass) but with differences in the extent of adhesion. For all the tested coupons, the OD value which represented the quantity of biofilm formation was 0 at 0 h. This indicated that there was no biofilm formed by *K. pneumoniae* on the fresh coupons. The reason behind was that the cells need time to adept to a new environmental conditions when the bacterial cells are newly transferred onto the fresh produce surfaces. Besides, the inculcated bacterial cells needed time to migrate on the produce surfaces to seek for suitable secure sites for adhesion [12-15].

Once attached to fresh coupons surfaces, they surrounded themselves with polysaccharides. These exopolysaccharide enabled the bacterial cells to attach to fresh coupons surfaces and also among them [1-12]. From data obtained and under different conditions, the OD value which represent biofilm density; increased with the increased incubation time but the increase reached a maximum at time 48h. Incubation time here can be known as contact time which is the time for the bacterial cells to contact with the fresh coupons surface [15].

Biofilm Formation and Substratum Effect

The catheter, stainless steel and glass surfaces and their effect on biofilm formation were investigated. The results showed that *K. pneumoniae* had adherence intensity on all experimental coupons as long as with experiment intervals as shown in figure 1.

Regardless of ecological conditions, the catheter coupons had a higher capability to attract bacterial cell and biofilm formation followed by glass surfaces while stainless steel was regard as a less attractive surface to bacterial adhesion and biofilm progression. Table 1 displays that a significant differences ($P<0.05$) between the densities of biofilm that formed on all substratum types that used in this study.

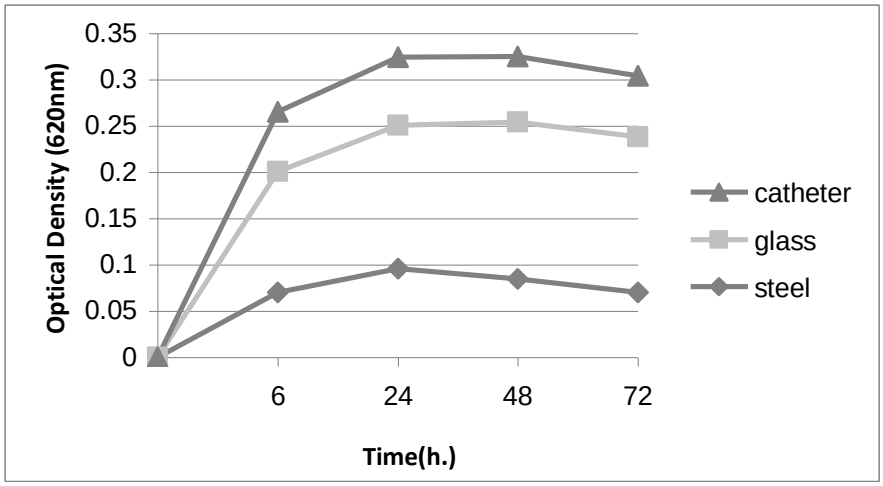

Figure 1- Relationship between substratum surfaces and mean values of biofilm formation by *K. pneumoniae* at 37˚C represented by OD 620nm.

The results of [11,14,15] that attributed bacterial attachment on coupons to surface properties are compatible with the results mentioned above. Therefore, the catheter showed a higher intensity of bacterial adhesion and biofilm progression compared to glass and stainless steel due to the physic-chemical properties of the catheter itself.

Hydrophobicity of bacteria and attachment surface are vital criteria in biofilm formation. Hydrophobicity of attachment surface can be influenced by the surface roughness. The surface hydrophobicity increases with the surface roughness due to Cassie effect. This Cassie effect occurs when the surface tension of a water droplet is supported by the rough bumps beneath it [16]. Donlan [4] reported that glass and stainless steel are hydrophilic materials while catheter represent as hydrophobic materials, and hydrophobic materials are reported as surfaces that provide a greater bacterial adherence [17]. On other hand, the glass surfaces presented a higher intensity of biofilm formation, compared to stainless steel coupons, which may be explained by the higher electric charge of the glass surfaces [11].

And surface roughness of the attachment surface is an important factor which can affect the removal of bacterial cells. The rougher is the surface, the more deep crevices or polish lines present on the surface. The high retention of the bacterial cells during rinsing process may be due to the possible entrapment of microbial cells in crevices of the surface, because these crevices provide refuge to the adherent bacterial cells from shear force [9]. According to the obtained data, the OD values for the catheter coupons were the highest comparing with OD values for the glass and stainless steel coupons.

Table 1- Effect of substratum surfaces and time on bacterial adhesion at 37 ˚C

Time (h.)	Surface type			LSD value
	Glass	Catheter	Steel	
0	0.00	0.00	0.00	0.00 NS
6	0.070	0.131	0.064	0.034 *
24	0.096	0.155	0.073	0.041 *
48	0.085	0.169	0.071	0.039 *
72	0.070	0.168	0.066	0.028 *
LSD value	0.035 *	0.051 *	0.026 *	----
* ($P<0.05$).				

This may indicate that catheter surfaces were the roughest attachment surfaces than other. Furthermore, there was a positive correlation between clean-ability (ability to be clean) and increased surface smoothness in the removal of biofilm. In the same surfaces type, the cleaner is the surfaces, the smoother attachment surfaces, as the result, less biofilm would formed. Thus, the properties of the attachment surfaces are important factors to determine the biofilm formation potential. The properties

such as surface roughness, cleanability, wetability (hydrophobicity) and vulnerability to wear influence the ability of bacterial cells to adhere to particular surfaces [17].

Conclusion

The results of our experiment conclude that this bacterium has the ability to adhere and subsequently form biofilm on all solid surfaces but in different degrees. In which, the catheter has significant higher attractive surface to bacterial cells and biofilm formation than glass and stainless steel surfaces. Therefore, the catheter surface regarded as most attractive surfaces followed by glass and stainless steel surfaces was the lowest.

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